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Molecular fragmentation of wheat-germ agglutinin induced by food irradiation reduces its allergenicity in sensitised mice

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ABSTRACT

WGA, an agglutinin from wheat germ which is largely responsible for many of wheat's allergies, was used as a model to investigate the action of ionising radiation on WGA's anti-nutritive effects in sensitised mice. Based on the molecular structure, the present study also examined the structural modification of WGA in relation to the range of dose. Structural integrity was monitored using HPLC, fluorescence spectrometry and circular dichroism. Results showed a loss of intrinsic activity and the formation of insoluble amorphous aggregates with a lack of native conformational structures after irradiation. Current findings suggest that the allergenic epitopes of WGA became less active and antigenic after high-dose radiation. The reduction of cytokines typical of allergic reactions, with decreased lymphocytic infiltrate, was observed in the gut of mice given irradiated versus native WGA. Food irradiation proved effective and safe in combating immunological and allergic effects of WGA.

1. Introduction

Lectins are proteins or glycoproteins which are widely distributed in crop plants and which have the ability to bind to cellular surfaces by means of specific glycol-conjugates (Breiteneder & Ebner, 2000). Because of this binding property, lectins are recognised as the major anti-nutrient of food. When consumed in excess by sensitive individuals, they can cause three primary physiological reactions: they can cause severe intestinal damage disrupting digestion; they can provoke IgG and IgM antibodies causing food allergies; and they can bind to erythrocytes causing haemagglutination and anaemia (Hamid & Masood, 2009). The anti-nutritional effects of lectins found in cereal grains are similar because they are closely related to one another both structurally and immunologically (Peumans & Cammue, 1986).

In wheat, gliadin, a component of gluten and the isolectin of wheat germ agglutinin (WGA), is involved in almost every acute and chronic inflammatory disorder, including neurodegenerative disease, inflammatory bowel disease, infectious and autoimmune diseases (Jones & David, 2005). WGA, the minor allergen from wheat, is a heat-stable protein and resistant to digestive proteolytic breakdown, which results in increased endogenous nitrogen losses and depressed growth rate in young animals (Cordain, 1999). In cereals, WGA concentrations range from 13 to 53 mg/kg. The highest WGA concentrations are found in wheat germ. The estimated quantity of total dietary lectin is in the range 0 ± 200 mg/person per day (Watzl, Neudecker, HaÈnsch, Rechkemmer, & Pool-Zobel, 2001).

Linear epitopes are a series of adjacent amino acids with no requirement for a particular secondary or tertiary structure, whereas a conformational epitope is strictly dependent on the folding of the protein chain (Restani et al., 2004). Because of their resistance to proteolytic breakdown, food allergens may facilitate the passage of undegraded conformational and linear epitopes into the systemic circulation, by their ability to increase the permeability of the intestine (Sjolander, Magnusson, & Latkovic, 1984). Therefore, compact three-dimensional structure, disulphide bonds and glycosylation, which may contribute to protein stability, are significant factors for the resistance of food allergens to routine food processing (Breiteneder & Mills, 2005a).

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Protein denaturation which involves structural or conformational changes to the native structure without alteration of the amino acid sequence has been obtained by various food processing techniques (Sathe, Teuber, & Roux, 2005). In these circumstances, however, only conformational epitopes are severely affected. Nevertheless, recent research has revealed the potential use of food irradiation as an alternative tool in reducing the antigenic characteristics of food allergens. The complete abolition of intrinsic activity and of molecular structure with the formation of insoluble amorphous aggregates has been a common finding after high doses of gamma irradiation (Vaz et al., 2011).

The safety and benefits of foods processed by ionising radiation are well recognised and over 30 countries use it for commercial purposes (Kume, Furuta, Todoriki, Uenoyama, & Kobayashi, 2009). However, our lack of knowledge of how a processed allergen can cause a problem, and of the analytical methodology with appropriate performance to trace it, is a reality. Thus, it is critically important to investigate the relationship between allergenicity and the stability of allergens after food processing, in order to help control the apparently rising tide of food allergies. Consequently, as a first step towards this, the main aim of the present work was to examine the effects of γ-radiation over a broad dose range on the molecular structure of WGA. We also investigated the degree of cellular infiltration in the gut and representative cytokines of Swiss albino mice sensitised and subjected to oral doses with irradiated WGA for 7 days when compared to non-irradiated WGA.

2. Materials and methods

2.1. Chemicals

The external fluorescence probe, 4,4′-bis-1-anilinonaphthalene 8-sulphonate (bis-ANS), and WGA (catalogue number L9640, highly purified) from *Triticum vulgaris* (wheat) were purchased from Sigma Chemical Co., USA. All solvents and other chemicals used were of analytical grade from Merck, Darmstadt, Germany. All solutions were made with water purified by the Milli-Q system.

2.2. WGA irradiation

WGA in phosphate buffer (pH 7.2) was lyophilised in borosilicate glass vials (16–125 mm) and then irradiated dry under an O_2 atmosphere by a Gammacell 220 Excel ^{60}Co gamma ray irradiator (Ottawa, Ontario, Canada) using doses of 1.0, 10.0 and 25.0 kGy at a rate of 8.25 kGy/h. Each dose was analysed after irradiation by the following methods.

2.3. Inactivation and unfolding

Haemagglutinating activity (HA), which was defined as the lowest sample dilution that showed haemagglutination, was evaluated as described by Correia and Coelho (1995). Specific HA (SHA) corresponded to the relationship between the HA and protein concentration measured according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA) as a protein standard in the range of 0–500 μg/mL. The percentage of the remaining SHA (%SHA_{REM}) was calculated according to the equation: $\%SHA_{REM} = (SHA)_{G_M}/(SHA)_{G_0} \times 100$, where G_M is the WGA SHA at each radiation dose (1, 10 and 25 kGy) and G_0 is the SHA of non-irradiated WGA (control).

To detect the nature of insoluble aggregates, the precipitate was treated with a chaotropic agent (8 M urea) and analysed by RP-HPLC after sample centrifugation. Irradiated samples were submitted to reverse-phase chromatography on a C-4 column (Vydac-Protein Peptide Ultrasphere – 4.6 × 150 mm, 5 μm particle size, 300 Å pore size) performed on an HPLC system (Shimadzu LC-10AD; Kyoto, Japan) and monitored at 215 nm. The column was equilibrated with 0.1% TFA in water (solvent **A**) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent **B**) in a non-linear gradient, where **B** = 0% at t = 5 min; **B** = 45% at t = 10 min; **B** = 50% at t = 30 min and **B** = 100% at t = 35 min.

Unfolding and aggregation of WGA was monitored by intrinsic fluorescence and light scattering using a spectrofluorometer (JASCO FP-6300, Tokyo, Japan). A protein concentration of 0.150 mg/mL, in 100 mM sodium phosphate buffer (pH 7.2) was used. The fluorescence emission intensity of tryptophan from irradiated WGA solution was measured at 25 °C in a rectangular quartz cuvette with a 1-cm path length. For intrinsic fluorescence measurements, the excitation was at 295 nm and emission was recorded from 305 to 450 nm, using 5-nm band pass filters for both excitation and emission. For light scattering measurements, the excitation was at 320 nm and emission was recorded from 300 to 340 nm. The light scattering was measured at 90° for the aggregation assays, obtained from the area under the fluorescence spectra. The hydrophobic surface was measured using the same conditions as employed for the intrinsic fluorescence experiment. Samples were transferred to a quartz cuvette and then mixed with 5 μM bis-ANS. The fluorescence emission spectrum was obtained from 400 at 600 nm, with an excitation at 360 nm (Bhattacharyya, Mandal, Banerjee, & Roy, 2000). The centre of spectral mass (CM) was calculated according to Eq. (1):

$$CM = \sum I_\lambda F_\lambda / \sum F_\lambda, \quad (1)$$

where F_λ stands for the fluorescence emission at wavelength I_λ and the summation was carried out over the range of appreciable values of F.

Far-UV CD spectra were recorded in the 190–250 nm region, in a 1-mm path length quartz cuvette using a spectropolarimeter (JASCO J-810). The instrument was calibrated with D-10-camphorsulfonic acid. The protein concentrations were as follows: non-irradiated (8 μM), 1 (8 μM), 10 (10 μM) and 25 kGy (20 μM) for WGA, in phosphate buffer, pH 7.2 at 25 °C. After irradiation the samples were centrifuged and the measurements were performed with the supernatant. The data were averaged for eight scans that were performed at a speed of 50 nm/min and collected in 0.5-nm steps. The baselines (buffer alone) were subtracted from the protein spectra. Results were expressed as mean residue ellipticity, $[\theta]$, defined as

$$[\theta] = \theta_{obs}/(10.C.l.n.),$$

where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 186 residues.

2.4. Sensitisation and oral challenge

Female Swiss albino mice (5 weeks old) were obtained from the breeding colony of the Departamento de Antibióticos da Universidade Federal de Pernambuco, Brazil, and given *ad libitum* access to food and water. The animals were kept in an environmentally controlled room, temperature 21 ± 2 °C, under a light/dark cycle of 12 h. Requirements for care and handling of experimental animals were according to international and Brazilian regulations. All test substances were administered intragastrically by tube. WGA was dissolved in 0.5 mL of 0.9% sterile saline.

Swiss albino mice were immunised subcutaneously on Day 0, 15 and 30, using 0.5 ml WGA (10 μg/mL) dissolved in saline without use of an adjuvant (five mice per group). Control animals were treated subcutaneously with 0.5 mL saline. Three days before starting the oral treatment in animals, they were stimulated with the same dose intraperitoneally. Over 7 days, mice were treated thus: group A, immunised mice were treated with 1 mL saline/day; group B,

immunised mice were treated with non-irradiated WGA; group C and D immunised mice were treated with irradiated WGA at 1 and 10 kGy, respectively. The dose of WGA (27 mg/kg body weight/day) was according to total dietary intake of lectins in human subjects consuming vegetarian diets (calculations based on data from Peumans & Van Damme, 1996).

2.5. Body weight and leukocytes evaluation

Body weight was determined before and after immunisation and after oral challenge. The final body weight of each group was obtained from the means of the individual values and expressed in grams. Blood samples were obtained and placed into micro-blood tubes containing the anticoagulant ethylenediaminetetra-acetic acid (EDTA). Haematological indices were determined by an automated particle counter, random-access clinical haematologic analyser Coulter STK-S (Hospital das Clínicas, UFPE, Recife, Brazil). Haematological indices were confirmed by optical microscopy, in which the morphology of leucocytes stained by the Giemsa method was observed.

2.6. Multiplex cytokine analysis

Multiplex cytokine analysis kits for mice were obtained by Genese Produtos Diagnósticos Ltda (São Paulo, SP, Brazil). Millipore multiscreen 96-well filter plates (Bedford, MA) were used for all multiplex cytokine kits. Assays were run in triplicate according to the manufacturers' protocol. Data were collected using the Milliplex Analyser 200 version 2.3 (Luminex, Austin, USA). Data analysis was performed using the software Analyst version 3.1. A four-parameter regression formula was used to calculate the sample concentrations from the standard curves.

2.7. Histology

After 7 days of oral challenge, the mice were sacrificed by decapitation. The histopathological evaluation of organs (jejunum) of animals was performed with an optical microscope. Fragments of organs were fixed in formalin (10%) and were subsequently dehydrated in a series of alcohols (70–100%), cleared in xylene and embedded in paraffin. Histological sections of 5 μm were

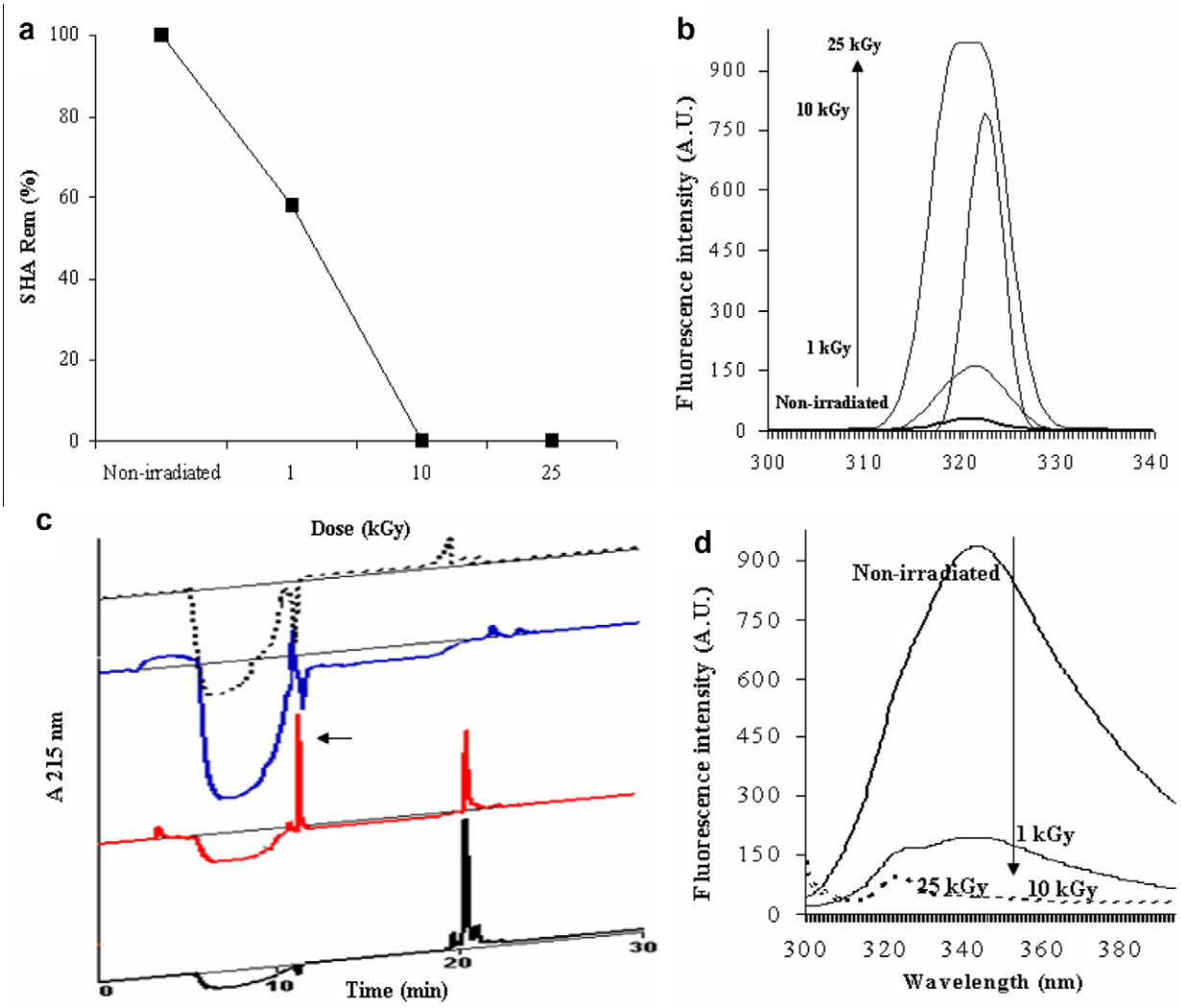

Fig. 1. Effect of γ-radiation on WGA. (a) The percentage of remaining specific haemagglutination activity, $\%SHA_{REM}$. (b) Light scattering for the aggregation assays; excitation (320 nm) and emission (300–340 nm). (c) Reverse phase chromatography by HPLC. (—) Non-irradiated and irradiated WGA at (—) 1; (—) 10 and (...) 25 kGy. (d) Intrinsic fluorescence; excitation at 295 nm and emission at 305–450 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stained by routine histologic haematoxylin and eosin (HE) and mounted between slide and coverslip with synthetic resin. After mounting, the preparations were evaluated using a video-microscopy system (MOTIC BA200 Microscope, digital camera with Motic 1000–1.3 M Pixel USB 2.0).

2.8. Statistical analysis

Differences between treatment groups of *in vivo* experiments were performed using nonparametric test (Kruskal–Wallis test) followed by the *post hoc* Dunns test in GraphPrism® (GraphPad Software Inc., San Diego, CA). Statistical significance was established at $p < 0.05$.

3. Results and discussion

We have previously shown that food irradiation induces a loss of intrinsic activity in food allergens (Vaz et al., 2011). This loss was completely dependent on the structural change elicited by molecular fragmentation, because the primary structure from irradiated allergens has been affected. For WGA, the change in the activity profile at each range dose is demonstrated in Fig. 1a. WGA is a homodimeric protein containing 16 disulphide bridges. The monomers associate with each other in a head-to-tail fashion forming a twofold symmetric globule. Each of the four carbohydrate-binding sites of WGA is located at the interface of two intercatenary domains (Muraki, Ishimura, & Harata, 2002). According to Pusztai et al. (1993), it is particularly worrying that detectable amounts of functionally-intact WGA are transported across the intestinal wall and may reach the systemic circulation, due to the heat stability and its resistance to proteolytic breakdown. However, as estimated, irradiation inhibited cell agglutination *via* WGA, which may affect its binding to the gut and reduce the allergic effect.

Since WGA exhibited a change in the activity profile due to intense aggregation, as detected by light scattering (Fig. 1b), the aggregate was treated with chaotropic agent (8 M urea) and analysed by RP-HPLC after sample centrifugation. Samples showed dissociation of the aggregates, suggesting that irradiation may induce peptide bond cleavage as well as complete fragmentation of the polypeptide chains at doses above 10 kGy. Non-irradiated WGA was separated by RP-HPLC into only one peak. The chromatography analysis revealed changes after exposure at 1 kGy, as indicated in Fig. 1c. The appearance of a previous peak (Fig. 1c – arrow) to the main peak indicates partial fragmentation of the WGA at this dose.

Conformational stability of WGA was investigated using fluorescence and CD spectroscopy. The shift in tryptophan fluorescence intensity and ellipticity at ~225 nm were observed with increasing doses (Fig. 1d and Fig. 2b). At a dose of 10 kGy, the protein possibly unfolds into non-native states that are prone to aggregation. Intense fluorescence due to bis-ANS bound to the WGA was observed at 10 and 25 kGy while 1 kGy shows significantly less binding (Fig. 2a).

Oligomerisation yields the basis for the multivalency necessary for typical lectin activities (Sharon & Lis, 1993). Therefore, any perturbation in protein structure that may affect the intrinsic activity after irradiation must be clarified. The positive band centred at ~225 nm in the far-UV CD spectra of WGA is characteristic of cystine residues immersed in an asymmetric environment (Drenth, Low, Richardson, & Wright, 1980). Its relatively elevated intensity is due to the high density of disulphide bridges, as well as the lack of secondary-structure repetitive elements. Radiation damage to sulphur-containing amino acids has been reported (Xu & Chance, 2005). This particular effect on disulphide bridges was observed in WGA and suggests that irradiation does not only compromise the dimeric structure but also produces a mixture of partially unfolded species at various stages of unfolding and large amorphous aggregates, after low and high doses of radiation, respectively. Such events were proven by the decrease of intrinsic fluorescence and high binding of bis-ANS to amorphous aggregates.

The current understanding about allergenicity of a plant food protein is determined by a sum of factors, including its abundance,

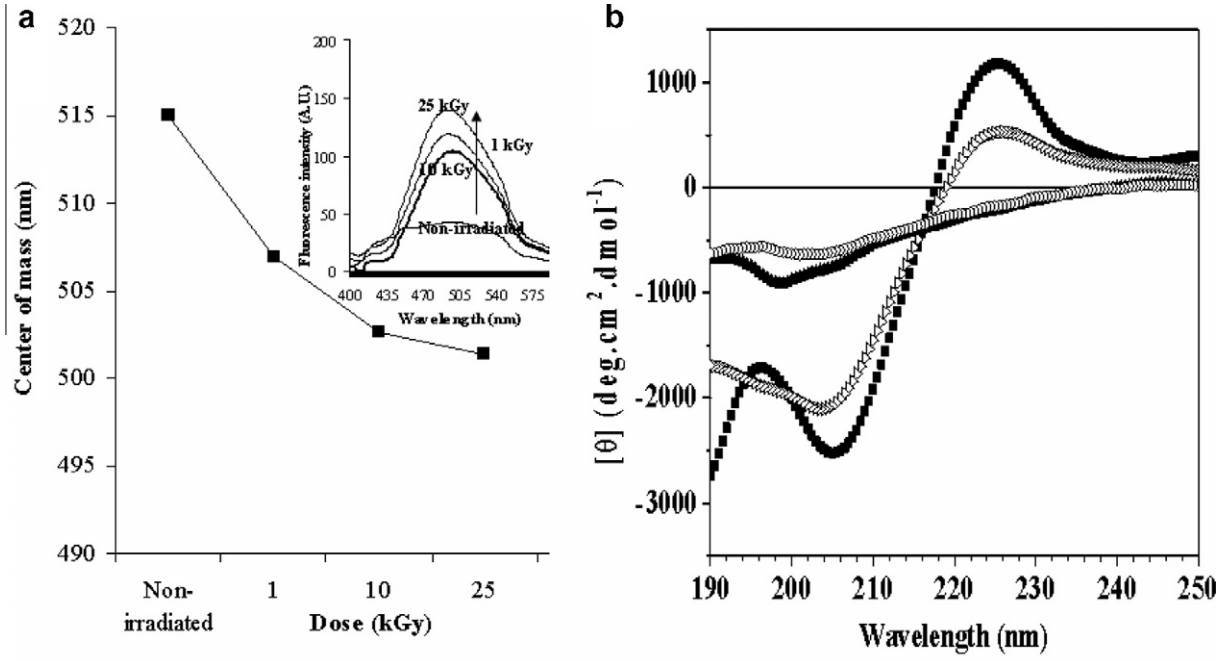

Fig. 2. Bis-ANS fluorescence and far-UV CD spectra of WGA. (a) Centre of spectral mass of bis-ANS fluorescence; excitation at 360 nm and emission at 400–600 nm. (b) CD spectra were measured in the far-UV range (190–250 nm) in 1-mm path length quartz cuvette. (θ) is given in degree squared centimetres per decimole. (■) Non-irradiated and irradiated WGA at (△) 1; (●) 10 and (○) 25 kGy.

Table 1
Body weight, leucocytes and cytokine profiles of mice challenged with native and irradiated WGA.

	Groups			
	G_0	G_1	G_2	G_3
Weight (g)				
Initial	21.6 ± 2.3	27.9 ± 1.0*	22.3 ± 1.3	37.7 ± 2.7*
Post immunisation	40.8 ± 1.1	35.9 ± 0.7*	35.0 ± 1.9*	33.3 ± 5.9*
Post treatment	42.9 ± 1.2	36.7 ± 1.3*	31.8 ± 1.3*,#	32.6 ± 5.1*,#
White blood count				
WBC[a]	1.7 ± 0.4	3.5 ± 0.3	8.6 ± 5.4*,#	5.8 ± 3.1
Large lymphocytes[b]	82 ± 6.6	88.2 ± 6.3*	90.6 ± 5.1*	92.2 ± 2.8*
Monocytes[b]	2.8 ± 2.0	1.6 ± 0.8	1.2 ± 0.8	1.3 ± 0.8
Neurophils[b]	12 ± 3.5	7.3 ± 4.0*	7.1 ± 4.7*	5.3 ± 1.9*
Eosinophila[b]	2 ± 1.2	0.4 ± 0.2	0.12 ± 0.1	0.8 ± 0.1
Cytokines and chemokines				
Eotaxin (pg/mL)	455.6 ± 70.3	1077 ± 74.5*	694.4 ± 1820.7	889.6 ± 298.8
IL-4 (pg/mL)	<0.31	23.2 ± 16.2*	12.4 ± 10.5	8.6 ± 4.4
IL-5 (pg/mL)	17.9 ± 4.2	191.6 ± 87.4*	23.1 ± 12.8#	45.4 ± 28.9
Rantes (pg/mL)	37.8 ± 8.7	68.1 ± 19.6	97.4 ± 46.7	69.9 ± 25.6

G_o, immunised control animals treated with saline; G_1, immunised mice were treated with native WGA; G_2,G_3, immunised mice treated with irradiated WGA at 1 and 10 kGy, respectively.

[a] White blood count in thousand per cubic millimetre.

[b] Mean per cent of total leucocytes.

* $p < 0.05$ compared to control.

# $p < 0.05$ compared to native WGA.

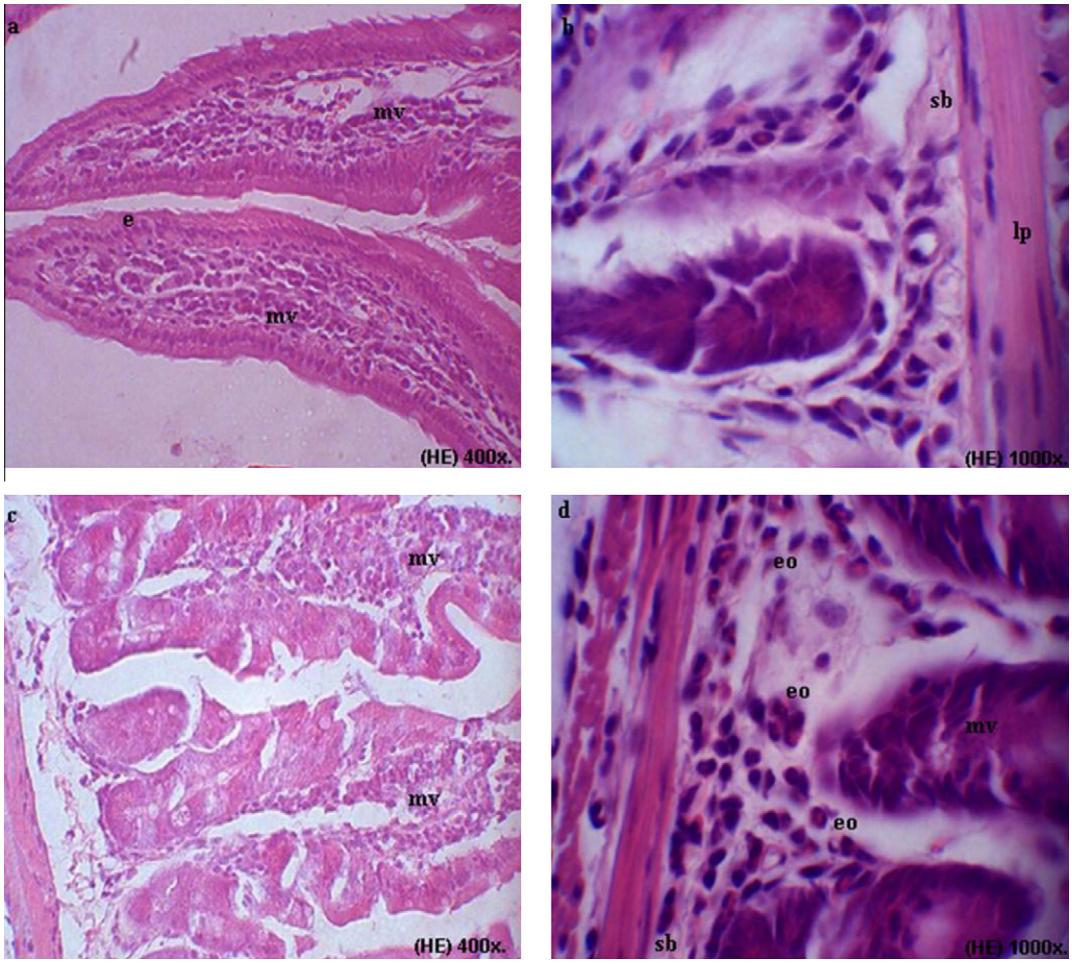

Fig. 3. Photomicrograph of jejunal mucosa of untreated and treated animals with native WGA. (a and b) Jejunum of immunised animals treated with saline: Note the microvilli (mv) coated high enterocytes (e) with a prominent striated cuticle. Central stroma with evident lymphocytic infiltration, submucosa (sb) well defined and preserved lamina propria (lp). (c) Jejunum of animal treated with native WGA: Note the moderate lymphocytic infiltrate filling the stroma of microvilli (mv). (d) Leucocyte infiltration in jejunal submucosa with numerous eosinophils (eo).

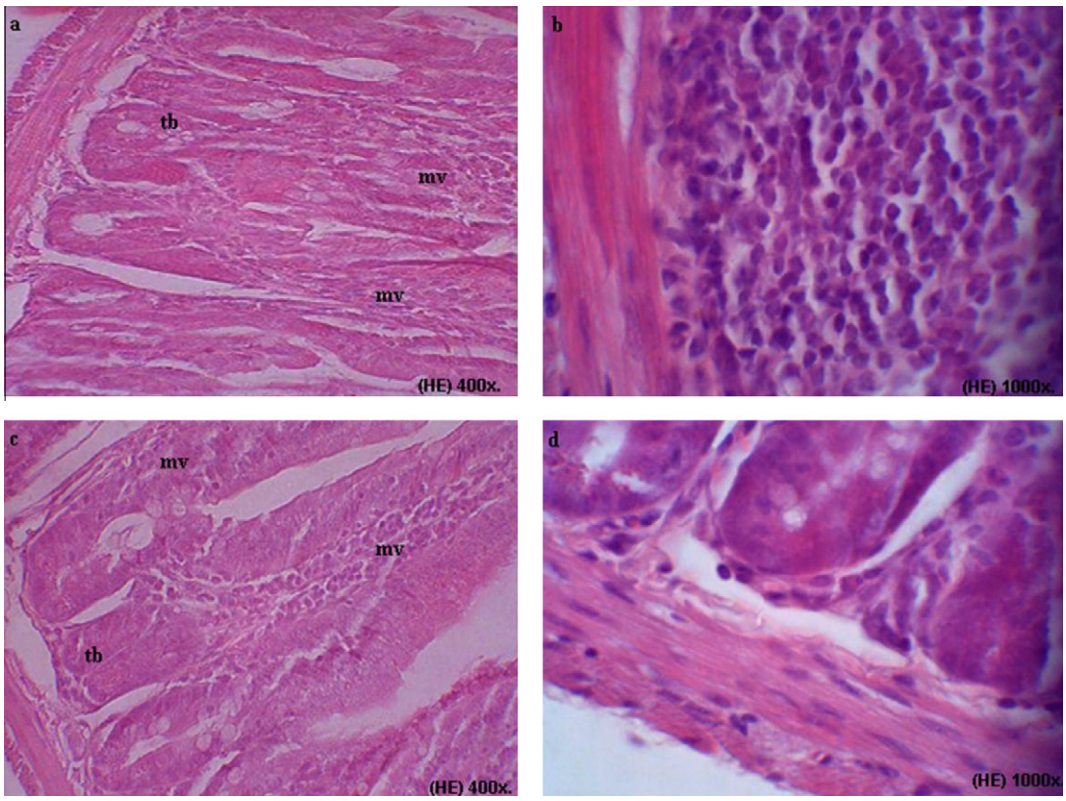

Fig. 4. Photomicrograph of jejunal mucosa of animals treated with irradiated WGA. (a) Jejunum of animals treated with irradiated WGA (1 kGy): Note the dense lymphocyte infiltration in the stroma of the microvilli (mv) and also around the tubular glands or crypts (tb) with numerous polymorphonuclear leucocyte (b). (c and d) Jejunum of animals treated with irradiated WGA (10 kGy) where the following can be observed: the same infiltration already mentioned for 1 kGy with a reduction of lymphocyte infiltration in microvilli (mv) and around the tubular glands (tb) when compared to previous treatments.

the stability to processing and digestion and the protective effect of food matrix (Breiteneder & Mills, 2005b). One aspect of food allergens that remains to be elucidated is the influence of the food matrix on the immune responses to food proteins. It has been hypothesised that the food body, consisting of fats, carbohydrates, and other proteins, may affect allergenic potential of proteins (Van Wijk et al., 2005). The main problem behind the conformational changes of proteins is that these are not always perceived by the general methods of analysis, which complicates the structural analysis if the food matrix is involved. However, these considerations should be investigated to clarify the contribution of food matrix to immune responses against irradiated food allergens.

In addition, since foods are often subjected to a variety of processing conditions, alteration in immunodominant epitopes may potentially affect protein allergenic properties. Processing may destroy existing epitopes on a protein or may generate new ones (neoallergen formation) as a result of change in protein conformation. More often, protein denaturation and/or modification to inactivate epitopes may be a more practical choice to reduce or eliminate food allergens after food processing (Sathe et al., 2005). Through gamma irradiation, we observed relevant functional and structural changes in a dose range above 10 kGy. Thus, we decided to investigate the anti-nutritive effects of irradiated WGA compared to non-irradiated samples. To study in depth the effect of irradiation on food allergenicity, we analysed weight loss, plasma levels of cytokines and leucocytes as well as the histological profile of the gut of animals sensitised and subjected to oral challenge with WGA for 7 days. All results are summarised in Table 1.

A significant ($p < 0.05$) weight loss of animals sensitised and given irradiated WGA was observed when compared with the control group challenged with native WGA. Although different, we note a greater weight loss for animals treated with WGA irradiated at 1 kGy. When blood leucocytes were determined (Table 1), we found increases of leucocytes and lymphocytes in mice treated with irradiated WGA at 1 kGy, being significantly different ($p < 0.05$) from the native-treated and untreated groups. The profile of cytokines revealed an allergic inflammatory response. Animals challenged with native WGA showed a significant ($p < 0.05$) increase of eotaxin, IL-4 and IL-5, when compared to the control group. The animals treated with irradiated WGA, which had weight loss and elevation of leucocytes, showed a significant decrease of IL-5, compared to mice treated with native WGA.

The histological profile of the gut of mice fed on diets containing native WGA was appreciably altered after feeding for 5 days. The jejunal mucosa showed moderate lymphocytic infiltrate filling the stroma of microvilli and a submucosa with numerous eosinophils (Fig. 3c and d) compared to non-immunised animals treated with saline (Fig. 3a and b). Jejunal mucosa of animals treated with irradiated WGA (1 kGy) showed dense lymphocyte infiltration in the stroma of the microvilli and also around the tubular glands or crypts with numerous polymorphonuclear leucocytes (Fig. 4a and b). In irradiated WGA (10 kGy), there was reduced lymphocyte infiltration when compared to previous treatments (Fig. 4c and d). Although we did not observe an association between WGA intake and body weight loss in sensitised animals treated with native WGA, certainly due to the short time of treatment, we can see that the body weight loss and lymphocytic infiltrate in the jejunal mucosa were reduced in the group treated with WGA irradiated at a high dose. Thus, endocytosis or binding to sub-epithelial tissues cannot be observed with irradiated WGA because the agglutination of cells has been abolished.

The increase of cellularity is common in a lectin-rich diet and has been observed (Pusztai et al., 1993). In accord with this, the number of polymorphonuclear leucocytes was higher with the

WGA-containing diet than with the control diet, possibly due to high secretion of eotaxin, IL-4 and IL-5 (Table 1). Eotaxin recruits Th2 cells that in turn produce IL-4 and IL-5, which help to amplify all their effects, resulting in the production of more eotaxin. Significant levels of eotaxin may result in eosinophil recruitment and degranulation, further Th2 recruitment, basophil degranulation and mast cell migration and differentiation (Gutierrez-Ramos, Lloyd, & Gonzalo, 1999). IL-5, acting as a chemokinetic factor for eosinophils, synergises with eotaxin in promoting the fast mobilisation of the eosinophil pool from the bone marrow (Bonecchi et al., 1998). Meanwhile, IL-4 stimulates the differentiation of CD4 + T-cells into Th2 cells, whereas its overproduction is associated with allergies (Tepper et al., 1990). Cell-mediated reactions are also involved, causing mucosal damage, such as crypt hyperplasia and villus atrophy in the late phase (Eigenmann, 2002; Ferguson, 1992). However, these events were not observed after 7 days of continuous challenge.

Although there was a considerable decrease in the allergic inflammatory response of animals challenged with irradiated WGA when compared with native WGA, the results of weight loss were not attuned to those obtained with cytokines in combating to anti-nutritive effects of WGA. However, the loss of intrinsic activity and insoluble amorphous aggregates with lack of native conformational structures was revealed after irradiation. This important finding may irreversibly impair linear and conformational epitopes, not only in the WGA, but also in other classes of food allergens, as observed in milk β-lactoglobulin, chicken egg albumin, and shrimp tropomyosin (Byun, Lee, Yook, Jo, & Kim, 2002). Studies such as ours, which are directed at understanding the mechanisms of food processing on food allergens, are scarce. Therefore, we investigated if food irradiation is safer and more effective in combatting clinical and immunological effects of food allergens. As our food supply becomes increasingly processed and complex, the stability of a protein to food processing may also be important in assessing its allergenic potential.

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